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FROM: AFIT/PA Security and Policy Review

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By: Capt Reid, H. Craighead, and T. Clark

CASE Number: AFIT/PA Case Number 020314

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Patterning Micro- and Nanometer Scale Lipid Bilayers Using a Polymer-Based Lift-Off

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Lipids, antibodies, antigen, avidin, and biotin have been patterned at nanoscale resolution for the precise immobilization and stimulation of immunological cells. We demonstrate that biomaterial can be patterned on silicon using a photolithographically patterned polymer lift-off technique. The nanoscale pattern is realized as the polymer is mechanically peeled away in one contiguous piece in solution. The 600 nm to 67 μm biomaterial patches provide a synthetic biological substrate for biochemical analysis. 100-nm unilamellar lipid vesicles spread to form a supported lipid bilayer on thermally oxidized silicon surface as confirmed by fluorescence recovery after photobleaching (FRAP). This method provides a new technique for immobilizing biomaterials, capturing antibodies from solution, and providing a platform for nanoscale antigenic stimulus.

Introduction

Over the past decade, micron-scale-patterning methods have been developed to immobilize functional biomolecules on silicon dioxide substrates for biosensor and bioassay applications. Many patterning techniques use lithographic methods, borrowed from microelectronics fabrication technology, to reproduce a mask pattern using biologically relevant chemicals (1). Photopatterning has been used to spatially distribute biomolecules, such as enzymes, antibodies, and nucleic acids, for the development of biochips on silicon, glass, and plastic substrates (2).

Recent lipid patterning techniques using microfabrication offer new methods for analyzing lipid mobility kinetics and for patterning biomolecules. The formation, stability, and sensitivity of lipid bilayers on solid substrates have been examined (3,4,5,6). Micro- and nanofrabrication-directed biomaterial patterning offers a greater control of cell analysis than bath application of biological materials. The microcontact printing process uses a poly(dimethyl siloxane) (PDMS) elastomeric stamp to pattern a wide array of chemicals, self-assembled monolayers (SAMs), and biomaterials (7,8,9,10). Microcontact printing has also been used to study many parameters of supported lipid bilayers, including lateral diffusion of lipid molecules (11), mobility of lipids within confined protein barriers (12), and electric fields have been used to induce concentration gradients in regions of spatially isolated lipid bilayers (13). Barriers to lipid bilayers have been formed on silicon with 10-nm thick gold corrals (14) and mechanical scratches (15). These research studies have used photobleaching experiments and scratching studies to demonstrate the fluidic nature of patterned lipid bilayers. These technologies make use of the lipid property of lipids that nonspecifically bind through water molecules on the oxidized silicon surface. Another study used spectral neutron reflection to characterize the structure of single lipid bilayers adsorbed to silicon substrates from aqueous solutions and confirmed adsorption with AFM analysis (16). While these are versatile and widely used patterning methods, they have the potential for surface fouling, nonspecific binding after primary layer application, and biomolecular denaturation from drying.

This paper presents a method for patterning biomaterial-conjugated lipid bilayers using a polymer lift-off method (17), as detailed in Figure 1. Vapor deposited di-para-xylylene, Parylene

C, is a conformal polymer film that adheres weakly to the surface, does not bind permanently to the substrate, and enables the patterning biomaterials in complex shapes with feature sizes below one micron. In this process, Parylene is deposited onto a substrate and photoresist is spin-coated over this layer. The resist is patterned using conventional photolithography and the sample is exposed to a reactive ion etch (RIE). The RIE etches the exposed regions of Parylene down to the substrate at 90 nm/min. When lipid vesicles are incubated on the patterned thermally oxidized silicon substrate the vesicles bind to the surface and form lipid bilayers on the regions of exposed silicon. After the lipids have incubated on the patterned surface, the sample is submerged into deionized water or buffer solution. The sample remains submerged in solution for the remainder of experimentation. Since neither drying nor compressive pressure are required in this patterning technique, delicate molecules conjugated to the lipids will not undergo denaturation or lose their functionality.

This technique is more suitable for biological experimentation than other conventional photolithographic lift-off techniques. Unlike other lift-off techniques, this method does not require photoresist as an underlying layer for chemical stripping or ashing to remove the resist, both which would destroy biological materials. The lift-off step simply requires a pinching of the Parylene in a corner with tweezers and a peeling away just as plastic wrap is removed from a flat surface. Parylene, having a chlorobenzene backbone, has similar non-sticking properties as Teflon and, subsequently, does not require additional surface treatment for its removal. The patterning process offers a reproducible method for patterning biomaterials without contaminating the substrate surface. The Parylene process allows biological material to be applied prior to the lift-off technique. The low permeability of the polymer prevents nonspecific binding during all steps prior to the polymer removal. Because the original patterning in the Parylene lift-off process is done by lithography, alignment to surface features can be done with high accuracy. The Parylene polymer is robust and can withstand acid, base, and ketone exposure. Here we demonstrate that this method can be used to direct the patterning of functionalized biomaterial templates of supported lipid bilayers from sub micron to millimeter resolution. Pattern distortions and lack of coating uniformity, which sometimes result from other methods, are eliminated in this process.

EXPERIMENTAL METHODS

Reagents. Milli-Q water is used for rinsing and dilution. OCG_OiR 897-12i photoresist is developed in Microposit MIF 300 solution. N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES), sodium chloride (NaCl), and phosphate buffered saline (PBS) (Aldrich, Milwaukee, WI) are used for buffer solutions. 0.1 μm Nucleopore polycarbonate filters (Whatman, Inc., Clifton, NJ) created 100-nm unilamellar lipid vesicles.

Silicon Wafer Preparation and Parylene Deposition. The wafers are cleaned in base and acid baths to remove surface contaminants. The wafers are baked at 1100°C for 50 minutes and annealed for 10 minutes in a wet oxide process to grow a 500-nm thermal oxide layer in the silicon substrate. Figure 1 details the fabrication steps used for the lipid parylene lift-off technique. A pinhole-free conformal layer of Parylene C is deposited onto 3-inch silicon wafers using the PDS-2010 Labcoater 2 Parylene deposition system (Specialty Coating Systems, Indianapolis, IN). The polymer thickness is dependent upon the amount of evaporated polymer. 1.5 g of Parylene C dimer is used to deposit a 1-μm thick Parylene film on five 3-inch silicon wafers.

Photolithography. 1.5 μm of photoresist is applied to the Parylene-coated silicon wafers. The samples are pre-baked for 3 minutes at 90°C and exposed using standard photolithographic techniques with a 10X stepper (Figure 1A). After development, the exposed portions of the Parylene film are subjected to an oxygen-based RIE step using the Plasma Therm 72 with an RF power density at 0.255 W/cm^2. This is illustrated in Figure 1B. After etching, the samples are dipped into a beaker of acetone to remove residual resist, rinsed with isopropyl alcohol, and washed in deionized water. The samples are then dried with a nitrogen gas stream.

Lipid Preparation. The lipids are purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine b sulfonyl (Rh-PE), 1,2-dipalmitoyl-*sn*-glycero-3- phosphoethanolamine-N-dinitrophenol (DNP-PE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) is zwitterionic and forms the backbone of the liposomes and supported lipid membranes. 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycerol) (2000) (DPPE-PE (2000) biotin), and 1,2-dioleoyl-*sn*-glycero-3-{[N(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl}(Nickel Salt) (DOGS-NTA). Lipids are dissolved separately and stored in chloroform at -20°C. Large unilamellar vesicles are prepared by extrusion (18). Lipids are mixed at desired molar ratios and dried to a thin film under a stream of nitrogen gas. 5-10 μmol of total lipid is dried per 13x100mm glass test-tube. The resulting films are placed under high vacuum for 1 h to remove residual organic solvent. Dried lipid films are hydrated in 10 mM HEPES, 140 mM NaCl, pH 7.4 using vigorous cortex mixing to a final lipid concentration of 2 mM. Following hydration of the multilamellar vesicle (MLV), suspension is subjected to 5 freeze-thaw cycles (liquid nitrogen/room temperature water). The MLV suspension is then extruded 10 times through two stacked 0.1 μm pore size polycarbonate filters using a high pressure 10 mL Thermobarrel Extruder (Northern Lipids, Vancouver, BC). Lipid vesicles are diluted with PBS, as desired. The functionalized lipid bound to the pattern on the silicon substrate is reacted with a target analyte as illustrated in Table 1.

Table 1. Functionalized lipid and target analyte association.

Functionalized Lipid	Target Analyte
DNP-PE	Alexa 488 Conjugated Monoclonal, mouse anti-DNP IgE
DPPE-(PEG)-2000-biotin	Alexa 488 Conjugated NeutrAvidin

Anti-DNP IgE Preparation. Monoclonal, mouse anti-DNP IgE molecules from the animals from the Cornell Veterinary School are purified and stained with NHS-Alexa 488 dye (Molecular Probes, Eugene, OR). The stock solution is diluted to 0.5 μg/ml, 7.4 pH.

Avidin Preparation. The sample is prepared by adding DPPE-(PEG)-2000-biotin lipids onto the patterned polymer surface. Alexa 488-conjugated NeutrAvidin (Pierce Chemicals, Rockford, IL) solution is diluted to 50 μg/ml in PBS, 7.4 pH.

Target Analyte and Lipid Application. A 20 μl drop of 2 mM lipid solution is placed on the Parylene-patterned substrate for 30 minutes, as illustrated in Figure 1C. Samples are incubated in 35-mm plastic Petri dishes (Fisher Chemicals, Pittsburgh, PA). The excess solution is washed off with Milli-Q water. The target analyte is applied onto the pattern and incubated for 30 minutes. After the incubation, the excess target analyte solution is transferred to a second Milli-Q water beaker in a 35-mm Petri dish and immersed in Milli-Q water. The Parylene is removed mechanically by peeling it off the substrate with tweezers under Milli-Q water like

plastic wrap off a flat surface. The polymer film is shed easily in one contiguous piece from the substrate. The resulting sample contains patterned lipids as illustrated in Figure 1D. The sample is observed in solution using epifluorescence microscopy on a Zeiss upright microscope with Omega Optical filter sets. Rhodamine fluorescent dye is observed with a 510-590-nm excitation/590-nm emission filter set and an Alexa 488 fluorescent dye is observed with a 450-490-nm excitation/520-nm emission filter set. Images are captured using a Spot CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI). The intensity readings of the fluorescent images of the patterned squares and lines are analyzed.

Fluorescence Recovery After Photobleaching (FRAP). FRAP testing is performed using a BioRad confocal head stage and an Olympus AX 70 inverted microscope. Photobleaching is performed with the 100X objective. The confocal laser light is focused an additional 10X at 100% power for a 1-minute exposure. Time series images are taken with a 10-second interval. The samples displayed full recovery of fluorescent molecules in the lipid bilayer after 30 to 180 seconds.

RESULTS AND DISCUSSION

Supported lipid bilayer formation offers a method for creating a model cell membrane template for analysis. Lipid polar head group characteristics allow them to form the lipid bilayers. Lipids are amphipathic, containing both a hydrophilic group and a hydrophobic group. This amphipathic nature allows the formation of the lipid bilayer. What allows formation of supported membranes is likely the interaction of the headgroups and the water film above the substrate. A thin film of water exists between the membrane and the substrate, as demonstrated by neutron scattering (19) and NMR (20) on glass beads. Therefore, lipid vesicles, which are closed bilayer membranes, have been shown to produce supported membranes on silicon dioxide substrates. 100-nm vesicles are employed in this study and have been shown to bind onto silicon surfaces and promote formation of supported bilayer membranes.

Figure 2 illustrates squares (with sub 600-nm widths on the lower right to 67-μm squares on the upper left) of POPC-/Rh-PE (99/1) lipid (2 mM) patterned on a silicon substrate using the Parylene lift-off technique. Sub 600 nm is nearly the lowest threshold attainable for patterning lipid bilayers using a 10X stepper. While this resolution is near the 10X stepper limit, an E-beam pattern could theoretically achieve patterns below 100 nm. The image is a fluorescent intensity profile of the patterned lipid molecules, one pixel wide, through the fluorescent image. A statistical analysis shows the average relative aerial density of fluorescently labeled lipids to the background is at least 150 times greater in the exposed regions than in the lift-off regions. Much of the observed background may be attributed to scattering of fluorescent emissions from the bright regions nearby. The actual contrast may be significantly better. Figure 3 is a higher magnification view of the second smallest patterned features, approximately 600 nm. The intensity profile shows the fluorescent intensity of the patterned lipid patches. The similarity in the peak profiles of each of the patches demonstrates the uniformity attainable with this submicron patterning technique as well as the low background signal between these patches.

Patterning antigen-conjugated supported lipid bilayers. Antibodies are molecules with high specificity for foreign antigens; this specificity directs their function in the immune system as accurate targeting molecules. Figure 2 shows the immobilization of DNP-conjugated PE in spatially defined locations. This figure illustrates the sharp resolution possible with this patterning technique. Once the lipid molecules have been patterned, the features are maintained

after the removal of the Parylene barrier. This offers the capability of subsequent chemical modification steps in the areas where the Parylene is removed, which is demonstrated here. 1 mol % Rh-PE is used in all the lipid applications as a liposome constituent so that the lipid bilayer pattern could be observed after polymer removal. Rhodamine is a robust dye that has minimal detectable photobleaching. Figure 2A illustrates the formation of 89 mol % DOPC supported lipid bilayer with 10 mol % DNP-conjugated PE and 1 mol % Rh-PE. Figure 2B illustrates the binding of the DNP-conjugated PE to the patterned lipid bilayer. Figure 2C illustrates layering of the molecules in this patterning process. Figure 2D illustrates the structure of the DNP-conjugated PE. This process serves as a model for targeting antigen on a patterned surface with labeled antibodies.

Patterning DPPE-(PEG)-2000-bioti-conjugated lipid bilayers.. The avidin and biotin molecules have a strong association constant, which Wilchnek, *et al.* (21,22) determined to be $K_a = 1 \times 10^{15}\ M^{-1}$ (21,23). For streptavidin to biotin the association constant was determined to be $K_a = 1 \times 10^{13}\ M^{-1}$ (22). Avidin-biotin technology offers an effective method for conjugating molecules in solution (23) and for immobilizing biomaterials on substrates (21). Avidin is a tetrameric molecule consisting of four homologous subunits that bind biotin (23). Avidin-biotin technology is a useful experimental tool since biotinylated molecules retain their biological, chemical, and physical characteristics after binding to immobilized or soluble avidin (22). These strong bonds survive conditions that normally dissociate weaker bonds. The strength and the versatility offered with binding biotinylated proteins, DNA, or polysaccharide make avidin-biotin technology widely applicable to biomaterial patterning (25). The avidin-biotin complex remains stable in conditions up to 132°C temperature, pH values between 2 and 13, concentrations of guanidine-HCl up to 8 M at neutral pH, and mild detergents (26). Figure 3 shows the patterning of a DPPE-(PEG)-2000-biotin. Figure 3A illustrates the formation of 89 mol % DOPC supported lipid bilayer with 10 mol % DPPE-(PEG)-2000-biotin and 1 mol % Rh-PE. The length of the carbon chain in PEG molecules offers a repulsive factor that reduces nonspecific binding. Figure 3B illustrates the binding of the (PEG)-2000-biotin to the patterned lipid bilayer. Figure 3C illustrates the layering of the molecules in this patterning process. Figure 3D illustrates the structure of DPPE-(PEG)-2000-biotin. This process serves as a model for patterning avidin on a surface for subsequent attraction of biotinylated molecules.

1.5 grams of Parylene polymer are used for the patterning of a batch of 5 silicon wafers. The polymer forms a film on not only the silicon wafers but also all the exposed surfaces inside the specialized Parylene deposition system. The majority of the polymer adheres to the liquid nitrogen trap that is used to cool and remove Parylene dimer vapor from the air in the oven before it returns the oven's pump. The resulting polymer film on the substrate is approximately 1 μm.

Uniformity of the final substrate relies upon photoresist and Parylene thickness, photolithography precision, RIE duration, and biomaterial incubation time. The ideal parameters for pattern formation of the submicron features are Parylene thickness of 1 μm and resist thickness of 1.5 μm. Use of thicker photoresist and Parylene reduces the homogeneity of sub-micron features. Parylene thickness below 1 μm would result in portions of the Parylene being etched away prior to sufficient pattern development. For homogeneous patterns, the photoresist must be applied uniformly without streaks. The exposure step requires accurate alignment, especially when a stepper is used. A focus exposure matrix is essential to determine the precise settings for patterning in the sub-micron range. The duration of the RIE step depends upon the

etching of the exposed polymer. If etching is terminated early, the smallest features will not be etched. If the etching is terminated to late, all the features will be larger than their original pattern and the Parylene will be too thin to peel away in one contiguous piece. Biomaterial incubation time is also an important factor for the generation of a homogeneous biomaterial surface. Additionally, the low objective use of the CCD camera (i.e. 10X objective for Figures 2A and 3A) lead to the appearance of a nonhomogeneous pattern. However, Figure 5 illustrates uniform submicron patterns observed with the 50X objective. Figure 5A illustrates the fluorescence intensity image analysis of 3 squares from a sample with features similar to those shown in Figure 2A. The analysis shows the relative aerial density of labeled lipids is at least 150 times greater in the exposed regions than in the lift-off regions. Much of the observed background may be attributed to scattering of fluorescent emissions from the bright regions nearby. (B) Fluorescence intensity image analysis of the 600-nm patterns from Figure 4. High signal to background contrast has also been attained at this nanoscale resolution for each spatially patterned lipid island.

Use of Parylene can be beneficial in the regions of both low or high concentration solutions. There is no upper limit to application time since the Parylene is covering the regions that need to remain unpatterned. Therefore, a long time period can be used to ensure maximal adhesion of the desired biomaterial. This is a benefit over other patterning methods, where increased adhesion time correlates with a linear increase in nonspecific binding. With a high concentration, the application can be terminated abruptly by rinsing without risk of redistributing the lipid to unmodified regions. Since the Parylene film covers the unexposed areas, surfactant or blocking solutions are not required to prevent nonspecific binding. The strong intramolecular forces of the patterned Parylene allow it to be removed as one contiguous piece from the substrate, exposing islands or lines of patterned lipid regions.

CONCLUSION

The Parylene lift-off technique offers a rapid and precise way to create supported micro- and nanometer-scale patterns. These patterns can be used to capture other biomaterials from solution, develop avidin platforms for subsequent biotinylated-molecule binding, and integration into biosensors, bioMEMS, and biological assay systems.

There are several advantages of this technique. The Parylene film removal can be performed at any step during the processing. Therefore, multiple reagents, such as biotin, avidin, and antibodies, can be added to the initial patterned lipid layer prior to Parylene removal. This would allow subsequent reagents to be added at high concentrations and maximal binding without concern for nonspecific binding on unpatterned surface areas. Parylene is biologically compatible polymer, provides a conformal coating with low permeability, and can be removed with a one-step mechanical lift-off. This technique permits the sample to remain submerged in solution so that the lipid bilayer is not disrupted from exposure to an air-water interface. Parylene does not have a permanent bond with the substrate, thus allowing easy removal in one piece. The conformal film of Parylene is pinhole-free, so no unwanted patterning occurs in unexposed regions.

Future research will replicate these experiments with a variety of lipids on silicon and other materials, such as glass and plastic. This technique offers a new versatile tool to pattern lipids and other biomaterial onto solid substrates with feature sizes below 600 nm.

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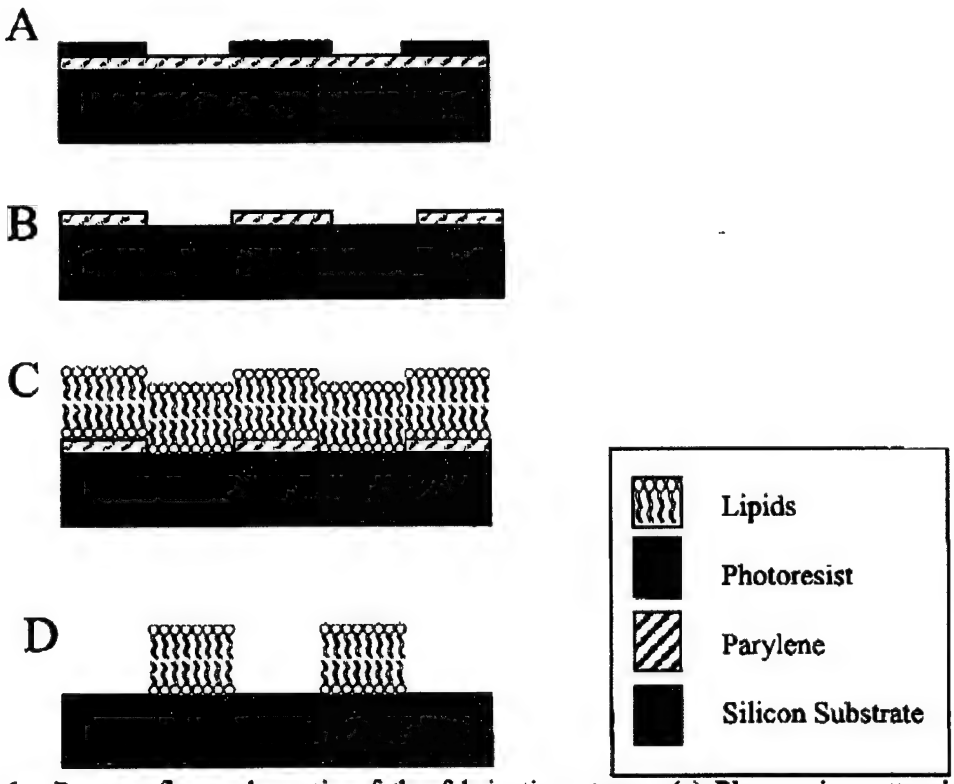

Figure. 1. Process flow schematic of the fabrication steps. (a) Photoresist patterning using optical lithography. (b) Reactive ion etching of Parylene and removal of the top photoresist layer. (c) POPC lipid immobilization. (d) Peeling of Parylene, resulting in a lipid bilayer.

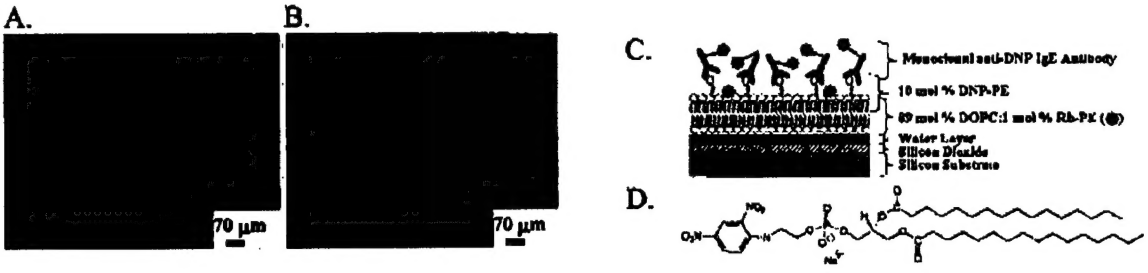

Figure 2. Optical fluorescence characterization of 500 nm to 50 µm squares and 1 µm to 20 µm lines of hapten-conjugated DOPC/Rh-PE supported lipid bilayer membrane. (A) Membrane composed of 10 mol % DNP-PE, 89% DOPC and 1 mol % Rh-PE. (B) The same pattern from Figure 2A with NHS-Alexa 488-conjugated monoclonal IgE. (C) Schematic of the lipid pattern illustrated in figures 2A and 2B. (D) Dinitrophenol-Phosphoethanolamine structure (27).

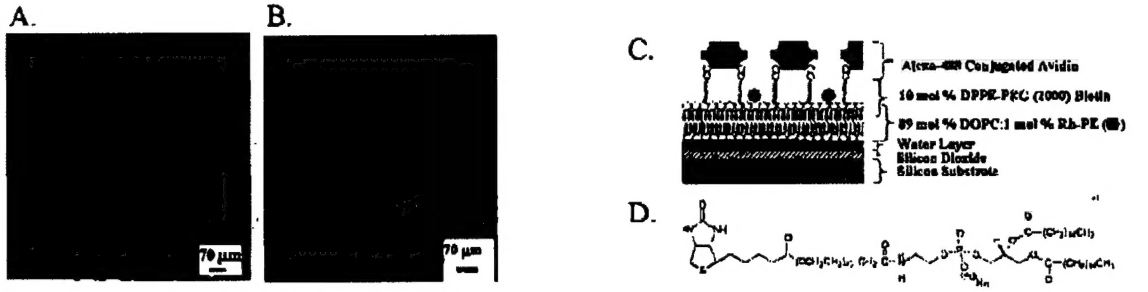

Figure 3. Optical fluorescence characterization of 500 nm to 50 µm squares and 1 µm to 20 µm lines of DPPE-PEG (2000) Biotin supported lipid bilayer membrane. (A) Membrane composed of 10 mol % DPPE-PEG (2000) Biotin, 89 mol % DOPC and 1 mol % Rh-PE. (B) The same pattern from Figure 3A with Alexa 488-conjugated avidin. (C) Schematic of the lipid pattern illustrated in Figures 3A and 3B. (D) DPPE-PEG (2000) Biotin structure (27).

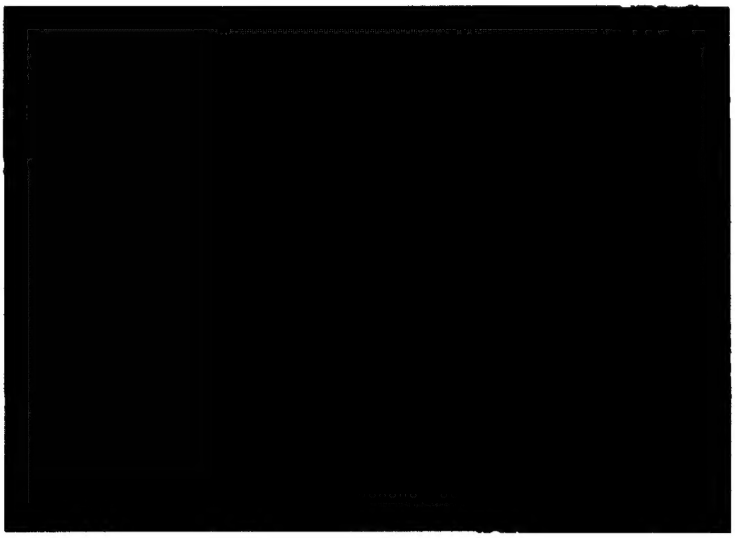

Figure 4. Optical fluorescence characterization of 600 POPC/Rh-PE (99/1) lipid (2 mM) (red) and 5% dinitrophenyl patterned on a silicon substrate (black) using the Parylene lift-off technique. Figure 3B shows 600 nm (left) and 1.1 μm (right) dots patterned in the same fashion (4.5 μm bar).

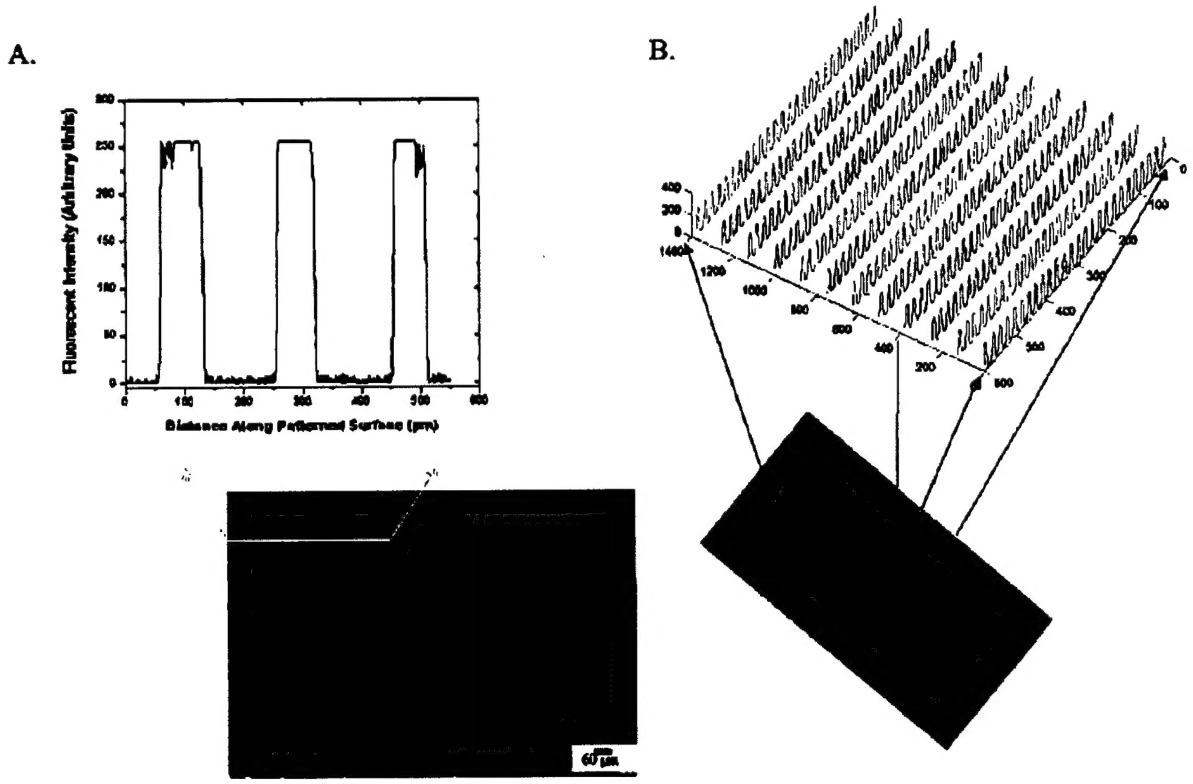

Figure 5. (A) Fluorescence intensity image analysis of 3 squares from a sample with features similar to those shown in Figure 2A. Note that a ratio of better than 150:1 in fluorescence emission has been achieved. (B) Fluorescence intensity image analysis of the 600-nm patterns from Figure 4. Note the high signal contrast of each spatially patterned lipid island.

Figure 6. Fluorescence.recovery after photobleaching (FRAP) illustration. Silicon wafer samples with a 500 nm thermal oxide layer were patterned with the polymer lift-off technique. A Bio Rad confocal imaging system and a 100X inverted microscope were used for the imaging. The laser light was focused for 60 seconds at a spot 10X smaller than the normal 100X field of view. Then a time series of images were taken at the normal 100X field of view. The images were taken at (A) 5 seconds (B) 35 seconds, and (C) 105 seconds after photobleaching. Scale bar in each picture is 5 μm. The yellow object in each of the lower right corners is a 50-μm Parylene line prior to removal. The Parylene is yellow since it has several layers of lipids attached and, subsequently, has a brighter fluorescence intensity.